# ALIZARIN AS AN INDICATOR OF BONE GROWTH

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## INTRODUCTION

Madder or its derivatives have long been used for the intra-vital staining of growing bone (Cameron, 1930). Alizarin, or its sodium salt, has more commonly been used during the past 100 years (Flourens, 1847; Gottlieb, 1914), and most workers have confirmed previous findings that only young or actively growing bone is stained red with the dye. Instead of giving alizarin orally, Gottlieb (1914) and Proell (1926), amongst others, gave sodium alizarin sulphonate—a water-soluble derivative—parenterally, and noted the rapidity of its action.

When given to a growing animal, alizarin stains the skeleton to an extent depending upon the dosage. Although young bones or parts of bones take up the dye better than the older parts, a single injection of a water-soluble compound in suitable amount can stain the whole bone system irrespective of age (Gottlieb, 1914). When a partly stained skeleton is studied in an animal sacrificed within a day or two of injection, the distinction between red (new) and white (old) bone is not always clear. It was this lack of precise definition between stained and unstained areas that led to the adoption of a technique in which the animal is allowed to survive for a period of days or weeks following administration of the dye: then the red bone (now 'old') is sharply contrasted with the new, white, bone. This technique followed naturally from the experiments of du Hamel (1739–43), of Hunter (1798), and of Macklin (1917), and was further elaborated by Brash (1924, 1926) and by Proell (1926).

Schour (1936), Schour & Hoffman (1938), Schour, Hoffman, Sarnat & Engel (1941), and most workers since, have used sodium sulphalizarate (1-2-dihydroxy-3-sodium sulphonic anthraquinone)—apparently identical with the sodium alizarin sulphonate used by Gottlieb in 1914. They gave it in a 2% solution in 0·45% sodium chloride by intraperitoneal injection, and reported that single doses of the order of 100 mg./kg. of body weight produced no significant toxic effects, though higher or repeated dosage did. Even single injections, however, gave a temporary retardation of gain in body weight in rats, with a subsequent return to a normal level. The dye was excreted in the urine and faeces, commencing within an hour.

Giblin & Alley (1942) did not find the madder method useful for measuring bone growth in the skulls of puppies. Madder feeding was too slow; intraperitoneal injections of alizarin too irritant (it is not clear whether they used insoluble alizarin or its soluble sodium salt) and purpurin gave poor results. They discarded the method. Nor did Robinson & Sarnat (1955) care for the alizarin technique. They said this about the use of madder (and, by inference, alizarin): 'Inclusion of madder creates an abnormal diet for the animals and may affect their growth pattern. The

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staining intensity varies with the age of the animal and the dosage of the dye used. *It cannot yield positive information regarding areas of absorption* [my italics]. Also, since resorption may lead to the removal of stained bone, vital staining will give incomplete data on the pattern of bone formation.'

Jarabak & Vehe (1951) and Jarabak (1951) confirmed that sodium sulphalizarate can stain bone irrespective of age if used in suitable concentration, but found that the method did not compare in sensitivity with that employing radioactive calcium, ($^{45}$Ca), although this isotope was shown to be concentrated in the same areas as the dye. Dixon & Hoyte (1959) have shown that the information obtained from a study of the disposition of radioactive calcium is very similar to that obtained from alizarin-stained specimens, and that the two methods are complementary.

The present position is that the use of alizarin in correct dosage as an intravital stain for growing bone is well attested; that the bone stained by it contrasts well with that formed subsequent to its administration; and that its distribution is similar to that of radioactive calcium. However, the dye is toxic; it may affect normal growth and by the very nature of its deposition it gives information only regarding bone deposition and not bone resorption, except by inference (Brash, 1934).

It is usually stated that the whole skeleton of a young animal is uniformly stained red with alizarin or madder (du Hamel, 1740, 1741; Gottlieb, 1914; Brash, 1924; Massler & Schour, 1951; Mednick & Washburn, 1956), though Strelzoff (1873), Macklin (1917) and Proell (1926) had showed histologically that the dye was distributed unevenly. Histological studies were limited to examinations of bones or of portions of bones, cleared or uncleared, under the low-power binocular microscope, or to ground sections of bones (the dye is calcium-fixed, and is lost on decalcification). Serial sectioning of undecalcified bones stained by alizarin was described by Roberts & Hoyte (1958), and the study of these sections confirmed the uneven distribution of the dye.

Since the results of serial sectioning of undecalcified bones in this context have nowhere been published, the opportunity is now taken of showing in detail the distribution of the dye, especially in the bones of the skull, of evaluating its usefulness and of establishing histological criteria which signify bone deposition, stasis and resorption.

## MATERIALS AND METHODS

Albino rats, rabbits and guinea-pigs were given a single intraperitoneal injection of a 2% solution of alizarin red (sodium sulphalizarate) at ages varying from birth to maturity, and were sacrificed at intervals following the injection. The dose was 50–100 mg./kg. of body weight (Schour, 1936). Gross specimens were prepared either by picking off the tissues after boiling in water, or by Green's antiformin technique (Green, 1934). Microscopic specimens were prepared from dried bones by using a high-speed carborundum cutting wheel (Hoyte, 1956, 1957), or from the whole heads of young animals by blocking in ester wax and sectioning with a special hard-steel knife on a sledge microtome. In this latter method (Roberts & Hoyte, 1958), 'Sellotape' was applied to the surface of the block prior to each cutting stroke. Serial sections varying in thickness as desired from 10 to 200 $\mu$ were obtained, each adherent to the 'Sellotape' (Pl. 1, fig. 1). The majority of the sections, cut at

100 $\mu$, were mounted and examined without further staining. Others were stained, after removal of the 'Sellotape', with haematoxylin and eosin; with toluidine blue (with and without decalcification on the slide); with Van Gieson's stain or with Masson's trichrome stain; or by the von Kossa silver nitrate technique. Microradiographs of certain sections, still on the tape, were prepared, as described by Miller (1954) and Graham (1955). These sections could then be mounted and examined for comparison with the microradiographs.

Most of the sections sufficiently unfragmented for examination by these techniques were thick—50–100 $\mu$—and therefore unsatisfactory for detailed histological examination of the soft tissues. For this reason, serial sections of the decalcified heads of control animals were prepared and stained in the usual way.

## RESULTS

### *Evaluation of the use of alizarin in the 'labelling' of new bone*

(1) *Rapidity of absorption and elimination*

Within 1 hr. of intraperitoneal injection of alizarin red into newborn animals, distinct coloration of the bones was observed. This colour appeared to reach its maximum in from 12 to 18 hr. In the fresh state it was reddish violet, but by the time the bones were prepared and dry, red was the predominant colour. The urine of the injected animals was seen to be coloured red within a few minutes, and was clear again within 24 hr.

(2) *Toxicity*

Variability of maternal care of the handled newborn animals made difficult an assessment of the immediate toxic effects of doses of the order of 50–100 mg/kg. However, weight records of the survivors showed that there was a temporary retardation of weight gain following injection, with a subsequent and speedy return to a normal rate of increase.

There is no doubt, however, that microscopic evidence of retardation of bone deposition can be found to confirm the toxic nature of the drug, even at these relatively 'safe' levels. In some rabbits injected on the first day of life, there was very little new bone formation even by the 5th day after injection, as seen by the virtual absence of new, white bone deposited upon the red. This is surprising, in view of the normal rapid increase in brain weight and of skull size in the neonatal period, and is to be correlated with the temporary retardation in weight gain. That it does not interfere with the usefulness of the dye as an experimental tool will be seen from the description given below.

(3) *Deposition of alizarin red in bone*

The dye is deposited in a single layer of bone, and indeed it is frequently apparent that it occurs in a single lamella. Even when given to newborn animals, in whom the entire skeleton appeared red, the microscopic picture revealed that there was still a deposit of red bone upon the previously existing, unstained, white bone (Gottlieb, 1914, quoting Strelzoff's observations; Proell, 1926; Hoyte, 1956). In certain areas of rapid growth, in the skull or at the epiphyses of long bones, whole trabeculae were coloured red. These bony bars were rarely more than one

or two lamellae thick, and were probably formed at one time throughout their thickness.

Diffuseness of staining may be due to several causes. It may follow from the slow and continued absorption of the dye after its administration—either from the use of madder root by mouth or from the formation of a tissue depot deliberately (in subcutaneous or intramuscular injections) or inadvertently (as in some of the present series of experiments). Thus, in some of the newborn animals, there was a gross escape of the dye at the time of injection, into the subcutaneous or extra-peritoneal tissues, seen when these animals were sacrificed in the early hours following its administration. Sections of the skulls of some animals, injected at birth and killed towards the end of the first week, showed, apart from the obvious red surfaces, diffusely pink trabeculae. The disposition of these showed them to be new, and it would seem that the alizarin was circulating for a longer time after the initial injection than one had supposed, and that in these too a tissue depot had been formed. (It seems unlikely that dye could be released by resorption from the skeleton in sufficient amount to effect appreciable staining of new bone in this way.)

Diffuseness of staining may, of course, be a false appearance, when seen by the naked eye or at low magnification, due either to the close aggregation of stained Haversian systems or of red trabeculae, to the red bone being seen through a thin layer of white, or to oblique sectioning. It may be a false appearance, due to the bone being cleared (or merely wet) prior to examination, when the clear distinction between red and white bone is lost. Paradoxically, clearing reduces the distinction between red and white bone in the 'red' areas, but increases the sharpness of contrast between these areas and adjacent, wholly white ones.

The dye is deposited on surfaces—subperiosteal, endosteal, on trabeculae flanking marrow spaces, around Haversian canals—known to be associated with the laying down of new bone. In sections counterstained with other dyes on the slide, the presence of osteoblasts in immediate contact with the newly laid down red trabeculae is evident (Pl. 2, fig. 9). In most of these areas, in specimens taken from animals surviving for longer periods, the red lines have been submerged by lamellae of white bone, where they have not been resorbed in whole or in part.

(4) *Microradiography of sections*

Microradiographs show that the red bone deposited on certain surfaces, especially that deep to the periosteum, is relatively translucent to X-rays (Pl. 1, figs. 2, 3). In the interpretation of these features in the microradiographs much care was taken to find areas in which there were no overlapping bone shadows to render the contrast between radio-lucent and radio-opaque bone too difficult. With this reservation, it is probable that the radio-lucent bone is recently formed and poorly calcified (Amprino & Engström, 1952; Amprino, 1953; Vincent, 1955). In animals surviving for longer periods, microradiographs of sections show that the red lines in certain areas have become more radio-opaque. They correspond now in general to areas of more highly calcified—older—bone, and are submerged in turn by more recently formed, radio-lucent bone. (It must be emphasized that there is no special feature of bone containing alizarin that distinguishes it on X-ray. It was thought that the injection might leave a 'mark' in the bone analogous to 'Harris's lines' (Harris,

1933), and support for this idea was suggested by the observations of Paff & Ecksterowicz (1950) and of Paff, Angulo & Ecksterowicz (1951) that alizarin prevents osteogenesis in tissue culture. However, no such 'mark' was found. One can only correlate the red lines on the actual sections with the trabeculae visible on the microradiographs of the same sections.)

(5) *Duration of staining with alizarin red*

When injected suckling or weanling animals were allowed to reach maturity before being sacrificed, it was seen that the red stain persisted in certain areas. The dye does not appear to be removed except in the course of normal resorption of bone.

(6) *Specificity of alizarin red for growing bone*

In the doses used, the dye did not appear to stain any bone other than new or growing bone. When injected into adult rats, it was still taken up only by bone developing at the epiphysial plate (in the albino rat the epiphyses do not fuse—Strong, 1925); at periosteal, endosteal and Haversian building sites (Hoyte, 1956).

*Histological findings in animals injected with alizarin red*

On examining the surfaces of the bones in the sections of the various skulls, different appearances were noted, depending on how long the animals had been allowed to survive between injection and sacrifice.

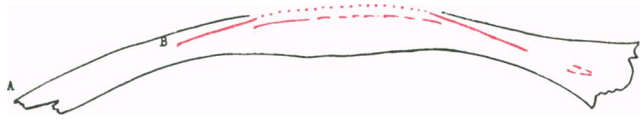
In skulls sectioned in the first few days following injection, the red-stained bone is that actively growing at the time of injection and circulation of the dye. Some areas presented red trabeculae deep to the periosteum, at varying angles to the surface. The appearance is one of rapidly spreading growth, and has been termed 'spreading trabecular growth' (Pl. 1, figs. 4, 5). In other areas the bone deposited deep to the periosteum consisted of a flat layer, varying somewhat in thickness in different situations. This is called 'flat subperiosteal growth' (Pl. 1, fig. 4). The presence of a completely stained surface implies that that surface was growing at the time of injection. Certain surfaces, or parts of surfaces, showed no stain at all. These were either static or resorbing areas, and in many areas of these young specimens no certain differentiation could be made between these two processes. The absence of stain does not necessarily mean, of course, that it has been removed—it may never have been deposited there. Sometimes, however, it is clear that one or other process is present. Stasis may be revealed by the absolute flatness of the unstained surface, resorption by the presence of scalloping (Howship's lacunae) (Pl. 2, figs. 7, 8). A resorbing surface is not usually completely unstained—there are usually red trabeculae or Haversian systems present. In general, the term employed in these early specimens is 'stasis/resorption'.

In sections from older animals, in which more time has elapsed between injection and death, both spreading trabecular and flat subperiosteal growth (of white bone upon red) can be seen. The difference between stasis and resorption is often more easily discerned. In the former, the static area may persist as an unchanged red surface, usually linear. In the latter, resorption is shown by the removal of red bone from areas where it previously existed in comparable sections; by the presence

of red lines 'disappearing' at a surface or at an edge (Text-fig. 1); or by an area which shows marked scalloping, with portions of red trabeculae or stained Haversian systems left (such an area may still appear wholly red to the naked eye). Sometimes an area of previous stasis or resorption is overlaid by new bone—whereupon a typical reversal line is seen at the junction of the two. No attempt was made to assess further resorption of any of the new, unstained bone laid down: it is preferable to get a continuity of information by varying the ages of injection and sacrifice, so as to assess always the white bone in relation to the red.

Text-fig. 1. Longitudinal section through intermediate third of parietal of a rabbit, injected at 18 days; survival 25 days; anterior edge to right. The inner outline represents the alizarin-stained bone, the outer new growth; . . ., represents surface resorption; – – –, the interruption of the alizarin lines by the diploe. ×27.

Text-fig. 2. Longitudinal section through parietal of a guinea-pig, injected at 8 days; survival 42 days; detail of coronal edge. Symbols are as in Text-fig. 1. ×36.



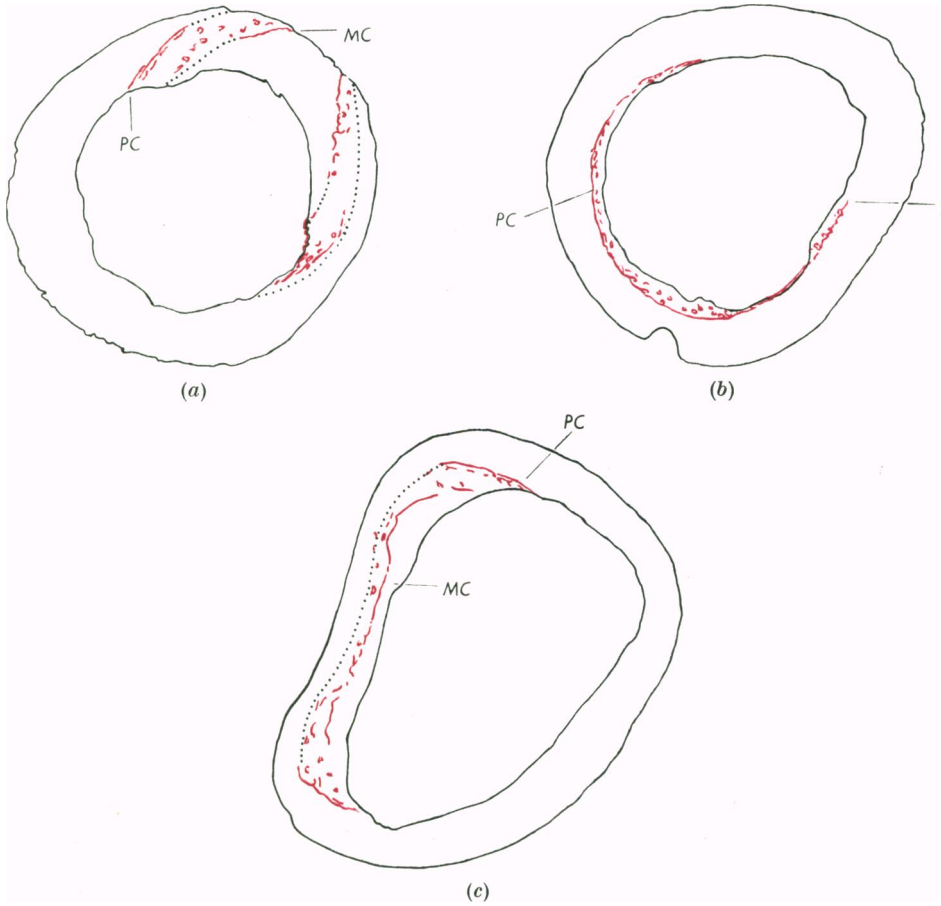

Text-fig. 3. Longitudinal section of parietal, through area of maximum convexity, in a rabbit injected at 1 day; survival 49 days; anterior edge to left. *A–B* is the apparent sutural increment. ×27.

In examining sections of the separate, dried bones from older animals, prepared on the carborundum cutting wheel, these and other criteria were recognized. In the bones of the skull vault red lines were seen marking the original inner and outer tables. Red trabeculae or occasional Haversian systems 'label' the area between the tables, and its subsequent development or removal could be traced. The inner and outer tables were sometimes joined together at or near the sutural edge by a 'closing' trabecula (Text-fig. 2). When seen, this can be taken as a fixed point. In its absence (Text-fig. 3), no conclusion can be reached as to the previous edge of the bone; no macroscopic view of the remaining red edge can be taken as a fixed margin; no measurements taken from such a 'margin' can be regarded as reliable.

In the long bones of these animals (Text-fig. 4) the fate of the red 'metaphysial cone' (Leblond, Wilkinson, Belanger & Robichon, 1950) and of the subperiosteal alizarin deposit was followed in serial transverse sections. Even if both lines have been resorbed, the bone which lay between them is clearly 'labelled' (as in the skull), and moreover is separated from a further layer of new bone deposited upon it by a distinct reversal line.

*Histological findings in control animals*

These criteria of surface changes in sections of alizarin-stained skulls and other bones were amply confirmed by the histological examination of control specimens. Spreading trabecular growth (Pl. 2, fig. 10) and flat subperiosteal growth (Pl. 2, fig. 11) are easily seen, and stasis or resorption can be recognized. Stasis is shown




Text-fig. 4. Transverse sections of the left humerus of a rabbit, injected at 25 days; survival 73 days. (*a*) is through the upper third, (*b*) through the middle and (*c*) through the lower third of the shaft. ..., represents a reversal line (deposition upon previous resorption); *PC*, the original alizarin-stained periosteal surface; and *MC*, the similarly stained endosteal aspect (metaphysial cone); at *X* the alizarin line disappears in the thickness of the bone. ×27.

by the absence—absolute or relative—of osteoblasts at a bone surface, or by the persistence of woven bone at one surface whilst subperiosteal lamellar bone is being laid down at the opposite aspect (Pl. 2, fig. 11). Woven bone was recognized in sections stained with haematoxylin and eosin by the uneven staining of its matrix and its patchy basiphilia (Ham, 1957), or, in addition, when of endochondral

origin, by the cores of calcified cartilage within many of the trabeculae. Reversal lines are frequently seen in specimens (Weinmann & Sicher, 1955).

Though recognizing that the role of the osteoclast in resorption is not finally settled, the whole experience here summed up of the comparison of alizarin-stained specimens with normal controls leads one to agree with Le Gros Clark (1958) that the presence of osteoclasts 'in areas of localized bone absorption... is so consistent, and the histological picture they present is so persuasive, that there can be little doubt of their participation in the removal of bone'. The presence of osteoclasts was deemed evidence of resorption, and they were found in areas interpreted as resorbing in the alizarin-stained specimens (Pl. 2, figs. 7–10; Pl. 1, fig. 6). Sometimes stasis and resorption existed side by side. Sometimes resorption and growth existed at a surface in very close proximity to one another (Pl. 2, fig. 12) (see also Kirby-Smith, 1933).

## DISCUSSION

The use of alizarin as a research tool in the study of bone growth has by no means been superseded, even in these days of the ready availability of radioactive isotopes. The ease and safety of its administration need no emphasis, and, in suitable concentration, it is not too toxic to be followed by normal growth. Like so many other techniques, it requires histological study, both in sections containing the dye and in sections from uninjected control animals. The preparation of sections of undecalcified bones is a problem common to all methods which employ substances bound to the inorganic constituents of bone, but it is not insuperable, as the above results, based upon a study of serial sections of bones so prepared, abundantly show.

Although many of the positive findings arising out of the study of gross specimens stained in life with this dye, or by madder root, have been known for 200 years, there was a need for a detailed assessment of their reliablity, based as they were upon a macroscopic distribution of the dye. Where exactly was the dye deposited, and how persistent was it? Were the lines of its deposition sufficiently clearly marked and permanent to be used for the measurement of growth? Were there indeed any fixed points in the growth of any bone?

On the negative side—that is, when considering bone 'subtraction' rather than bone 'addition'—could this method of intra-vital staining give reliable information about the sites and extent of bone resorption? That this *is* so follows from a consideration of the results listed previously, and will be further elaborated in later communications. It has been insufficiently recognized that the mere presence of red coloration in a bone might signify deposition of bone, stasis or even resorption, and therefore many workers (amongst them especially Massler & Schour (1951) and Mednick & Washburn (1956), who repeated some of Brash's (1924, 1934) work on the braincase of the infant pig) totally misconstrued surface changes. No measurements which rely upon the distribution of alizarin in bones can be considered sound unless microscopic evidence of that distribution in similar bones can be directly compared.

The great drawback to the use of alizarin in studies of bone growth is the difficulty of recording, and hence of publishing, the results: only in colour can the full value of such studies be realized. However, given a reliable qualitative assessment of the location of the dye, and of changes in bone surfaces in relation to its sites of deposition,

it should be possible in a series of similar bones to show by measurement the amounts of new bone added and the directions of growth.

It can be concluded that alizarin red, given by intraperitoneal injection to growing animals, in doses of the order of 50–100 mg./kg. of body weight, on a single occasion, has a distinct sphere of usefulness:

(i) It is rapidly absorbed, rapidly fixed in the growing bone and the excess rapidly excreted.

(ii) It is not so toxic as to prevent subsequent normal growth in relation to its sites of deposition.

(iii) Its staining effects are precise, and limited to a single layer of bone being laid down at the time of its circulation in effective concentration.

(iv) The lamellae in which it is deposited show no abnormality to X-rays; nor does its presence prevent the continued normal calcification of these lamellae during growth.

(v) It persists in the lamellae of bone which it stains until removed by the normal processes of resorption.

(vi) By its use areas of resorption can be readily recognized, and, provided histological sections can be prepared, precisely delimited.

(vii) With it, areas of stasis can be shown, and by the judicious selection of a graded series of injected animals, 'fixed' points can be ascertained. Thus the patterns of bone growth may be followed, and measurements may be made with some degree of reliability.

## SUMMARY

Alizarin red was given by intraperitoneal injection to growing animals, in doses of the order of 50–100 mg./kg. of body weight. Its distribution was studied in serial sections of the undecalcified skulls and limb bones. It is shown to be an intra-vital stain of some precision, rapidly absorbed, fixed only in the bone forming during the time of its circulation in effective concentration, and removed only in the course of normal resorption. Growth of the animals after injection, temporarily retarded, quickly became normal; and subsequent histological and microradiographic appearances were normal also.

The different appearances of the red-stained surfaces are noted, and criteria suggested that enable deposition, stasis and resorption to be recognized. These appearances were confirmed by the examination of sections from decalcified controls. Alizarin, used in this way, therefore, can give precise information about patterns of bone growth and resorption, and may permit of exact measurements of these activities.

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## EXPLANATION OF PLATES

### PLATE 1

Fig. 1. Cutting undecalcified sections from an ester wax block. A 'Sellotape' ribbon is seen.

Fig. 2*a*. Microradiograph: transverse section of interfrontal suture in a 2-day-old rabbit injected with alizarin at birth. A core of radio-opaque bone runs through the frontal. ×36.

Fig. 2*b*. Drawn from the same section. All surfaces are red, the centre white. The spreading trabeculae are red, new and relatively radiolucent. The dense core consists of older, unstained bone. (The unbroken line —— represents flat subperiosteal growth; ||| or × × × spreading trabecular growth; and ··· stasis or resorption.) ×27.

Fig. 3*a*. Microradiograph: transverse section of frontal bone from the same rabbit, near front of orbit. Note the sharp, 'punched out' appearance of a resorbing area, and the radiolucent border superiorly (flat subperiosteal growth). ×36.

Fig. 3*b*. A drawing of the same section (symbols as above). ×36.

Fig. 4. Same animal as in fig. 2. Transverse section of mandible, inferior border. There are red trabeculae spreading medially (to left of photograph), and a flat, red subperiosteal layer laterally (right). ×36.

Fig. 5. Same animal as in fig. 2. An area of spreading trabecular growth, entirely red, cut transversely at the anterior extremity of the maxilla. Note the 'fading' of the red trabeculae into the tissues. ×36.

Fig. 6. One-day-old control rabbit. Detail of area shown in Pl. 2, fig. 10. Osteoclasts are seen. Haematoxylin and eosin. ×216.

### PLATE 2.

Fig. 7. Same animal as in fig. 2. Transverse section of mandible, showing spreading trabecular growth on the lingual aspect (right), scalloping on the crypt surface. ×36.

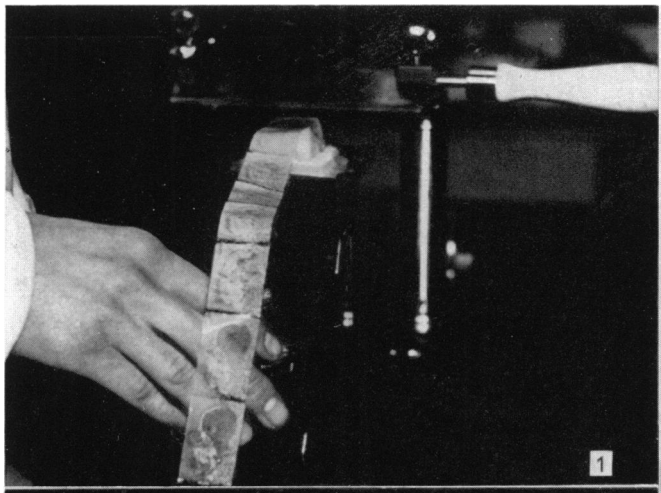
Fig. 8. Detail of lingual wall of crypt. ×90.

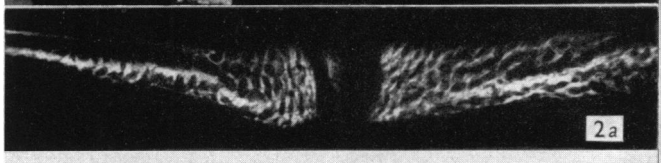
Fig. 9. As in fig. 8; counterstained with Toluidine blue. Note the intense periosteal reaction on the lingual surface and the presence of osteoclasts (arrowed) laterally. ×90.

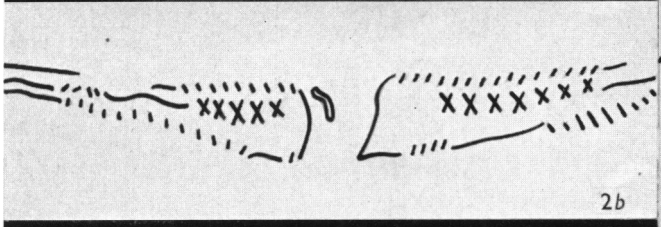
Fig. 10. One-day-old control rabbit showing an area similar to the preceding. Note osteoclasts in the tooth crypt. Haematoxylin and eosin. ×90.

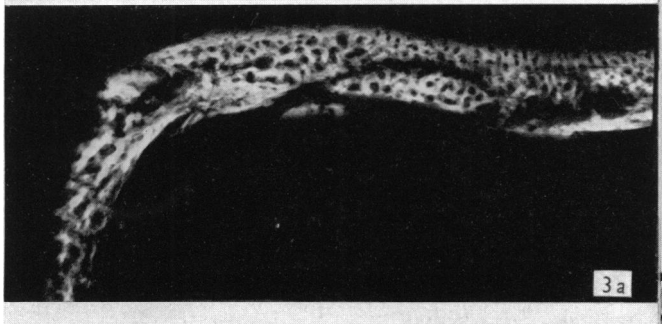
Fig. 11. Six-day-old control rat. Horizontal section of the parietal bone, just behind the coronal suture. There is flat subperiosteal growth externally (left), with numerous osteoblasts; and relative stasis endocranially (right), with few osteoblasts and persistent woven bone. Haematoxylin and eosin. ×216.

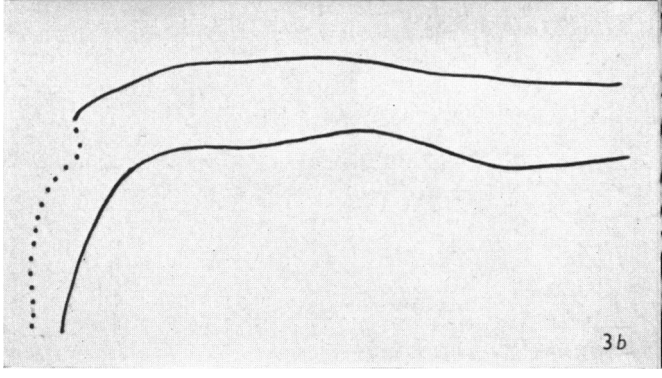
Fig. 12. One-day-old control rabbit. Transverse section of frontal bone, in medial wall of orbit. The osteoclasts lie on the orbital (left), the osteoblasts on the endocranial aspect of the thin plate. Masson's trichrome. ×216.

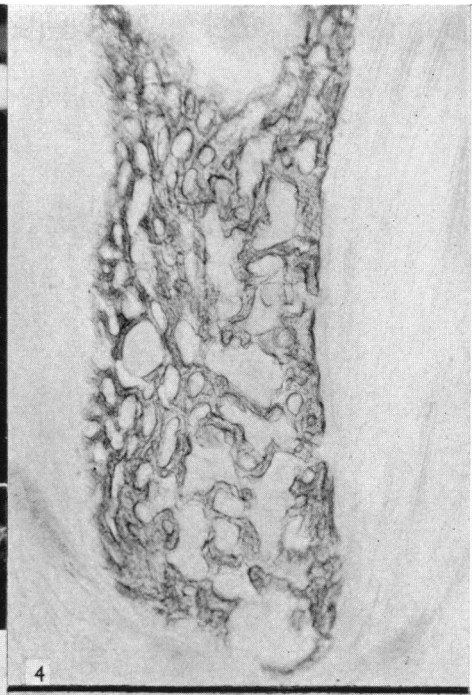


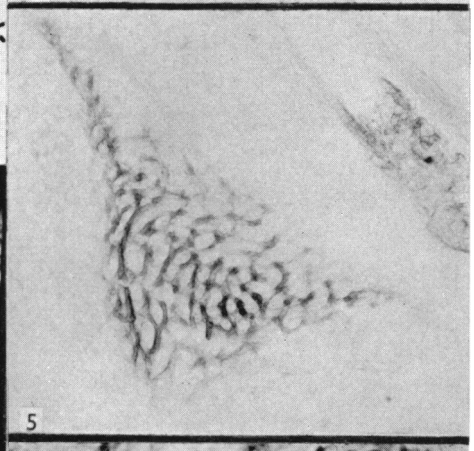



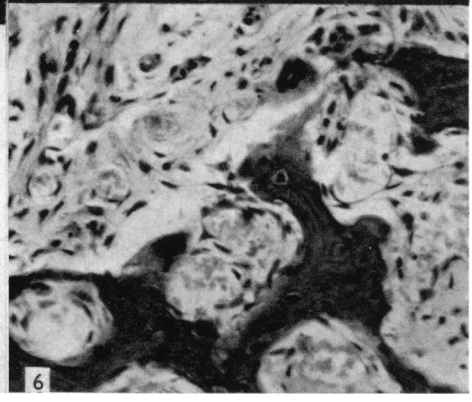




HOYTE—ALIZARIN AS AN INDICATOR OF BONE GROWTH

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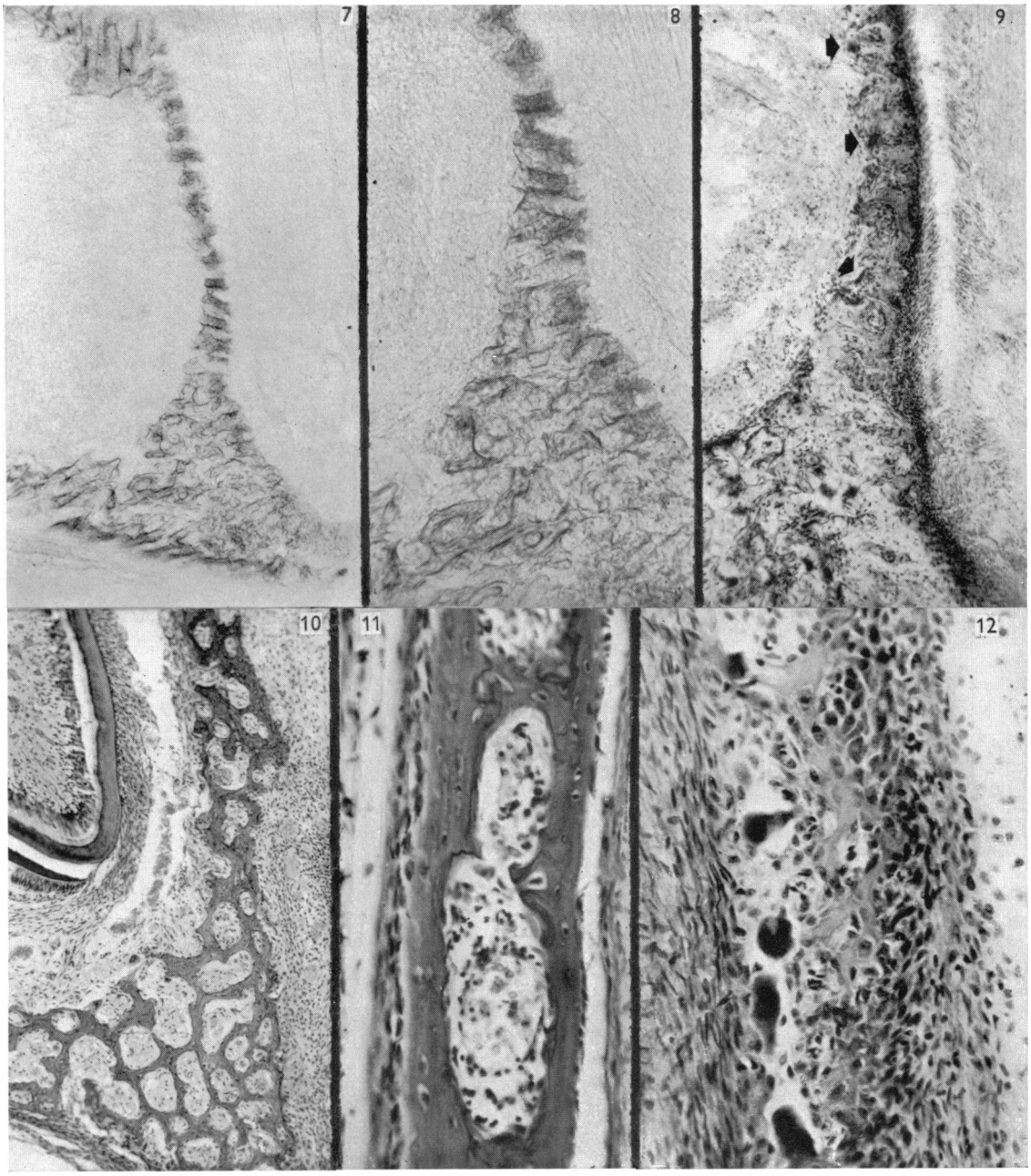


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